CHRONICALLY ELEVATING SPONTANEUOUS ACTIVITY IN CULTURED NEURONAL NETWORKS RESULTS IN DISTINCT HOMEOSTATIC CHANGES

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LIST OF SYMBOLS AND ABBREVIATIONS

MEA	Multi-electrode array
DIV	Days *in vitro*
ASDR	Array-wide spike detection rate

SUMMARY

During the first few weeks *in vitro*, cultured neuronal networks exhibit spontaneous bursts of action potentials, even in the absence of external input [10]. Pharmacological intervention, as well as electrical stimulation provide a direct means for chronically elevating this spontaneous network activity and allow the study of homeostatic plasticity [3, 8]. In this study, embryonic day 18 rat cortical neurons were dissociated and grown on multi-electrode arrays (MEAs). During the third week *in vitro*, activity was chronically increased for 24 hours by disinhibiting the network using a $GABA_A$ receptor antagonist, or by electrically stimulating the network using the MEA. Spiking activity was continuously recorded through the MEA before, during, and after each perturbation, and the frequency of spontaneous population bursts was obtained to give insight on how the overall network was affected. Chronic pharmacological blockade of $GABA_A$ receptors increased culture burst frequency and after washing out the drug, a significant homeostatic reduction in bursting was observed. Meanwhile, the same increase in elevated burst frequency was attempted using distributed electrical stimulation. Although electrical stimulation did not increase activity in every trial, of the cultures that did experience elevated bursting, a surprisingly distinct homeostatic effect was seen. When compared to drug treated cultures that had similar increases in burst frequency, there was a significant difference in bursting immediately following treatment ending. These experiments may help improve our knowledge of clinical interventions where neural tissue is subject to chronic electrical stimulation, as well as identify the consequences of these therapeutic approaches.

CHAPTER 1

INTRODUCTION

The goal of this thesis is to utilize multielectrode arrays to investigate neuronal network activity during and after chronic increases in spontaneous bursting. Bursting was chronically increased in one of two methods: 1) Adding the inhibitory receptor blocking drug bicuculline and 2) Electrically stimulating through electrodes in the MEA. The purpose of this research is to better understand how neurons can adapt to changes of activity.

Homeostatic plasticity is the term used to describe the mechanisms in both single neurons and neuronal networks that help regulate overall average levels of activity. This is a complex process that is thought to involve changes in receptor density, amount of neurotransmitter release, synapse sensitivity/responsiveness, and levels of excitatory/inhibitory feedback. Homeostatic plasticity is an extremely important response in that it allows the human brain to survive in times of great irregularity. For this reason, much more is needed to be learned concerning the mechanisms of homeostatic plasticity.

It has been previously observed that following chronic increases in spontaneous activity, cultured networks exhibit a period of rest where bursting is reduced or even diminished [3]. It is unclear, however, if this decline in bursting occurs independently of the method of activity increase.

Among the multiple methods of increasing culture bursting, drug incubation and electrical stimulation were utilized in these experiments. Multielectrode arrays allowed for the simultaneous recording and stimulating of neuronal cultures *in vitro*. This method of studying neuronal networks produces a broad spectrum of ways to investigate homeostatic plasticity.

CHAPTER 2

LITERATURE REVIEW

2.1 Homeostatic Plasticity

Many of these mechanisms of homeostatic plasticity are thoroughly explained in the Turrigiano & Nelson Nature Neuroscience Review "Homeostatic plasticity in the developing nervous system" [7]. In this review, the effects of acute bicuculline treatment are explored. It is noted that after 2 days in bicuculline, a culture undergoes a change that resets the activity back to normal, pre-drug levels. Additionally, this review states that when raising culture activity for two days feedback excitation is reduced and feedback inhibition is increased onto pyramidal neurons. It is important to note that the mechanisms used in regulating homeostatic plasticity are believed to be different depending on the type of perturbation of the network.

Goel and Buonomano [3] explored the effects of drug incubation and chronic electrical stimulation on rat cortical brain slices in their 2013 study. Following the forced increase of activity, pyramidal neurons in the slices were recorded from intracellularly. Among other interesting results, this study showed that the administration of a $GABA_A$ receptor antagonist and bursts of electrical stimulation at 100Hz both induce a post treatment decrease in the frequency of spontaneous activity events.

Goel and Buonomano's work raised the following questions: "What homeostatic changes occur during activity elevation?" and "Do similar results appear if stimulation is reduced to the same frequency of which bicuculline treated cultures are bursting?"

2.2 Electrical Stimulation

To address the questions raised above, multielectrode arrays assisted in the stimulation of plated cortical neurons. In the past few decades, electrical stimulation has been used extensively to alter activity in cultures and brain slices. Methods can be used to

quiet bursting and lessen their interference when studying plasticity in cell spiking [9]. In Wagenaar, Madhavan, Pine, and Potter's paper "Controlling Bursting in Cortical Cultures with Closed-Loop Multi-Electrode Stimulation" [8], different stimulation protocols were explored and levels of bursting were quantified. It was found that stimulation onto single electrodes at low frequencies (<0.1 Hz) increased bursting in the culture. Biphasic 400μs phase width pulses were shown be most effective here. Wagenaar et al. did what many similar studies don't do and recorded network activity during stimulation. They found that low frequency stimulation evoked bursts time locked to the stimulation pulses with latencies of about 50-200 ms.

Additionally, Bologna et al. showed in 2010 that 0.2 Hz, biphasic 200μs pulse width stimuli administered for 3 minutes twice a week for the life of the culture produced an increase in bursting rate in the 30 minutes following stimulation [2]. This is an interesting result, but like many similar studies, lacks the reporting of activity during stimulation; meaning, we do not actually understand the culture during stimulation.

CHAPTER 3

DESIGN AND METHODOLOGY

3.1 Cell Culture

The Multi Channel Systems multielectrode arrays (MEAs) used in these experiments contain six individual wells with nine electrodes in each; this allows the simultaneous recording of six separate sister cultures. The electrodes are 30μm wide and arranged in a 3x3 grid, 200μm apart. Prior to plating, all MEAs were sterilized, coated in polyethyleneimine (an attachment promoting polymer), washed, and coated in laminin (an axon growth substrate). Figure 1 from Appendix A shows that neurons touch the electrodes they are plated upon and that, in as short as one day, axons begin to connect the network.

Day 18 embryonic rat brains were carefully dissected to isolate only the cerebral cortices. Dissociation was then performed using papain (an enzyme that catalyzes the breakdown of proteins) and DNase (an enzyme that catalyzes the cleaving of phosphodiester links in DNA). Neurons and glia were plated onto the 6-well MEAs at a density of about 3000 live cells per microliter and at a volume of 12 microliters. Cultures existed in a growth medium containing serum that was partially replaced every three days. The environment was maintained at 35°C, 5% CO_2, and 65% relative humidity and the MEA was covered in a Teflon lid that is permeable to air but not fluids [1]. More about this plating procedure can be found in the Potter and DeMarse paper [4]. During this experiment, three of the six wells were experimental and the other three were kept as controls.

3.2 MEA Electrophysiology

Continuous recording of extracellular action potentials were obtained for each electrode in the MEA. Signals were filtered, amplified, and then recorded using an open-

source, real-time electrophysiology interfacing system called NeuroRighter [5]. All recordings were performed in an incubator under the same conditions previously mentioned.

3.3 Drug Treatment and Electrical Stimulation

Bicuculline is a $GABA_A$ receptor antagonist that disinhibits network activity. GABA, the main inhibitory neurotransmitter in the mammalian brain, regulates the amount of excitability in the system. Without the normal function of GABA, activity increases drastically. 20µM concentrations of bicuculline were introduced into the cultures at 17-18 days *in vitro* (DIV). This remained for 24 hours until all wells (treated and control) were washed four times. The number of culture wide spontaneous bursts in one hour were counted in order to find the change in frequency before and after bicuculline treatment.

Electrical stimulation of the network was implemented using 0.75 volt, biphasic, positive first, 400 microsecond width pulses at 17-18 DIV and lasted for 24 hours. The pulses followed a Poisson distribution onto random electrodes within the MEA well. The frequency of pulses was determined by the normalized increase in bursts that was obtained during the bicuculline experiments.

3.4 Analysis and Statistics

Because all action potentials were recorded a time histogram was created by grouping spikes into 10 millisecond bins. From here, population bursts stand out by having a larger number of spikes per bin. Burst were counted if they reached a threshold of 150X baseline firing rate and if a previous burst had not been recorded in the past ten seconds. By using this method, each culture had a unique burst threshold that took into account its specific level of activity.

Each culture's array-wide spike detection rate (ASDR) was plotted using custom written MATLAB code. As mentioned earlier, the histogram function was used to sum the number of recorded action potentials that occurred in a 10 millisecond bin. The

number of bursts reaching threshold in one hour were counted and a burst frequency was determined.

Bursts were counted for every hour of every culture and then were normalized by dividing by the culture's average baseline burst count. This gave percentages where 100% represented no change from baseline burst rate. Burst were analyzed by four periods: 3 hours pre-treatment, 24 hours during treatment, 3 hours immediately following treatment, and 21-24 hours following treatment. Next, bicuculline treated cultures and electrically stimulated cultures were divided by the average of their respective controls in order to show activity changes independent of culture fluctuations.

A non-parametric Mann-Whitney U test was used to compare differences between sets of burst rates. The goal here was to show that both bicuculline and stimulation increased bursting significantly when compared to controls. Additionally, it was important to show that there was no significant difference between bicuculline treated burst rates and electrical stimulated burst rates. Lastly, average burst rates for the 3 hours immediately following treatment were compared between bicuculline treated and electrically stimulated cultures.

CHAPTER 4

FINDINGS

4.1 Results

When comparing spontaneous activity of the control cultures and the cultures treated with bicuculline, there was an increase in the frequency of population bursts by an average of 458% ± 92% of average baseline frequency (n=9). Immediately following washes, spontaneous population bursting was completely diminished. One day later, some but not all bursting returned to an average of 41% ± 25% of the baseline rate. When observing firing rate as opposed to bursting rate, there was an average of 105% ± 50% of baseline spiking immediately following washes, indicating that although there was no bursting, spiking was still occurring. The ASDR of one culture and its control is shown in Figure 2, Appendix A, along with normalized average bursting rates over time.

This same increase in burst rate was attempted to be applied to other cultures electrically through the electrodes of the MEA. With the stimulation protocol used, not every culture displayed an increase in bursting rate. When averaged together, a 146% increase from baseline rate was seen (n=14). As this is not comparable to the bicuculline treated cultures, three successful trials were pulled from the data to be analyzed separately. The average increase of these cultures was 189% ± 11% during stimulation, 88.5% ± 22% immediately following stimulation, and 162% ± 23% one day after stimulation. Figure 3(A) shows an electrically stimulated trial that successfully increased bursting while 3(B) and 3(C) show normalized activity over time. When analyzing why stimulation did not reliably increase network activity in every trial, it was noticed that many stimuli evoked “mini-bursts” that did not reach the set threshold for the particular culture. On average, the probability that a stimulus evoked a burst that met threshold was 24.5% ± 5%. This information can be seen in Figure 3(D).

Next, because the successful electrically stimulated trials were not significantly similar to the average of all bicuculline trials, three of the most similar bicuculline treated cultures were chosen to allow further comparison. The normalized bursting rates over time for these cultures can be seen in Figure 4(A). These cultures were normalized to their respective controls to eliminate normal activity deviation in the culture. A statistical test was then performed on each time period to show differences in burst frequencies. The normalized burst rates were similar during treatment ($p>0.5$) and significantly different immediately following washes and the ending of stimulation ($p<0.05$). One day after treatment bicuculline and stimulated cultures showed similar changes in burst rate ($p>0.5$). This information can be seen in Figure 4(B).

4.2 Discussion

The results above would not have been possible without the use of multielectrode arrays allowing the continuous recording both during and after stimulation and drug treatment. It was shown that a 24-hour $GABA_A$ receptor blockage disinhibited the network and induced a homeostatic decrease in burst frequency following washing the drug out. Unfortunately, electrical stimulation did not reliably increase network activity in every trial. Because only 24.5% ± 5% of the stimulus pulses delivered to a culture actually evoked a burst, a change in stimulation protocol could help increase overall network bursting.

Of the bicuculline treated and electrically stimulated cultures that experienced comparable activity elevation, there was a significant difference in bursting levels following treatment. This suggests that homeostatic changes are dependent on the method of activity perturbation. More research is needed to explain the exact mechanisms that are at work during each type of homeostatic response.

CHAPTER 5

CONCLUSION

Deep brain stimulation (DBS) is often used in clinical practices to treat anything from depression to Parkinson's disease. Electrodes are implanted within the brain and controlled amounts of electrical pulses alter the patient's motor or sensory functions. Despite the use of this procedure, little is known about its underlying mechanisms. More research in the field of neuro-electrophysiology will greatly benefit both doctors and patients of disorders that could be treated using DBS.

Bicuculline treatment and electrical stimulation are often used for *in vitro* studies when the investigator wants to see a more spontaneously active culture. This can then be used to model an epileptic system [6]. My research will assist in knowing what consequences come from each form of activity manipulation.

5.1 Future Directions

In the future, the development of a closed-loop electrical stimulation protocol will be useful in precisely matching the bursting rate produced by pharmacological disinhibition. This will allow the study of how spiking patterns can be influenced by distinct stimulation parameters. The first area of modifying stimulation parameters will be changing pulse width and exploring the effect on the probability that a stimulus will evoke a burst. In addition, an investigation of synapses, the junction where one neuron acts upon another, will be beneficial in explaining the results found in the experiments previously performed.

APPENDIX A

FIGURES

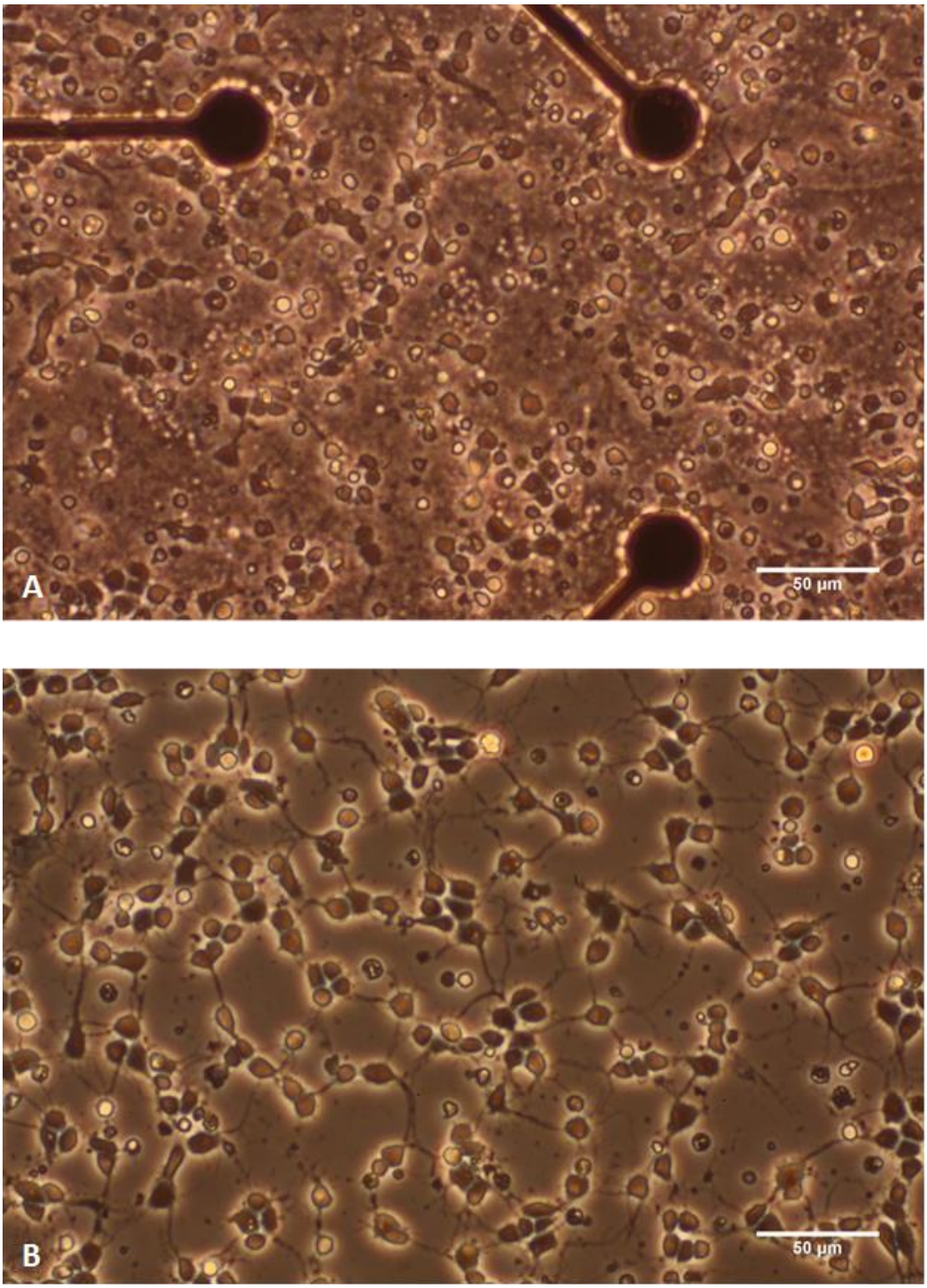

Figure 1. Dissociated neurons platd onto an MEA. Taken at 1 DIV. (A) Neurons surround electrodes. (B) Axons form connections between neurons.

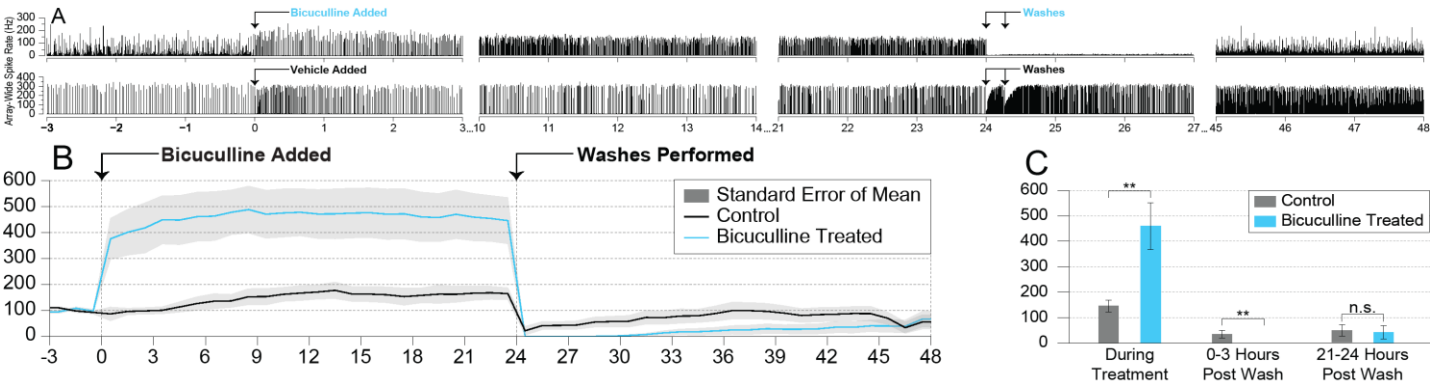

Figure 2. (A) *Top*, Array-wide spike histogram of a 16DIV culture treated with bicuculline for 24hrs. During bicuculline treatment, burst rate was raised to 185% of its baseline level. Bursting was completely depleted following washes, but later returned. *Bottom*, Spike histogram of a 16DIV sister control culture. Washes were also performed at t = 24hrs. Bin size = 1s. (B) Average % of pre-bicuculline burst rates over time. Activity was normalized by culture and then averaged. Shaded areas represent SEM. (C) Normalized average activity for 24hrs during bicuculline treatment, 3hrs immediately following washes, and 3hrs one day after washes. Bars represent SEM. **p<0.01 (Bicuculline n=9, control n=8)

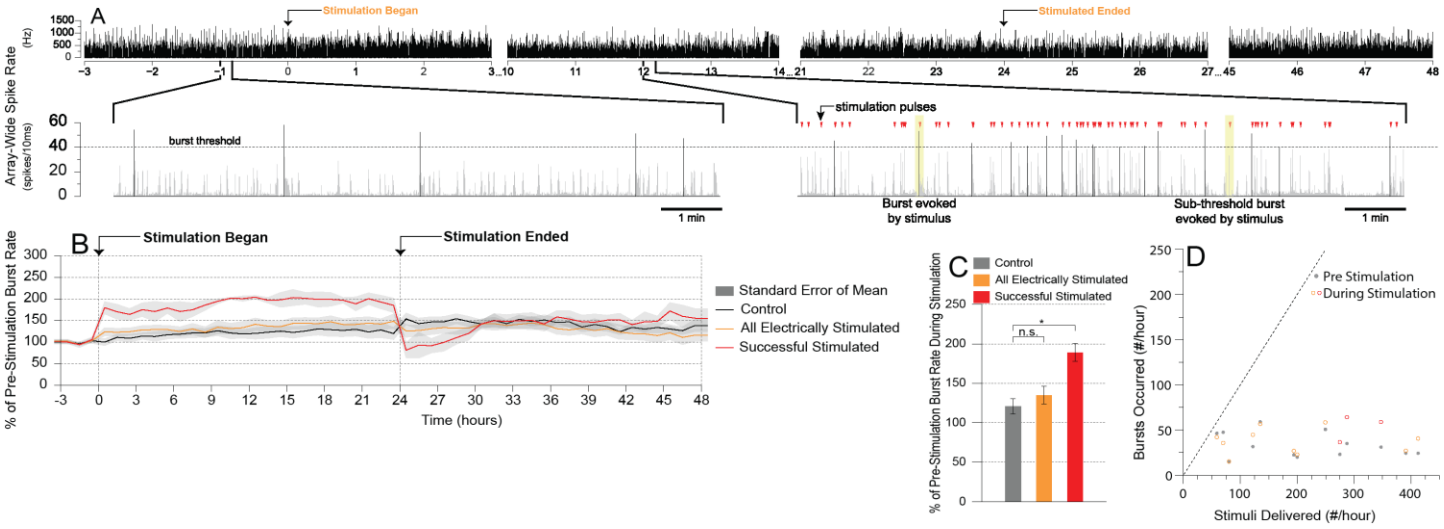

Figure 3. (A) *Top*, Array-wide spike histogram of a 17DIV culture receiving electrical stimulation for 24hrs. Bursting was raised to 203% of its baseline level. Bin size = 1s. *Bottom*, 10min portions of spike histogram are shown. Bin size = 10ms. (B) Average percent of pre-stimulation burst rates over time. Activity was normalized by culture and then averaged. Shaded areas represent SEM. (C) Normalized average activity for 24hrs during electrical stimulation for controls, all stimulation trials, and selected trials showing significant increases. Bars represent SEM. *p<0.05 (D) Stimulus rate versus evoked burst rate for each culture. Dotted line represents equal stimulus and evoked burst rates. The average probability that a burst occurred due to a stimulus is 24.5% ± 5%. (Electrical n=14, control n=12, successful electrical n = 3)

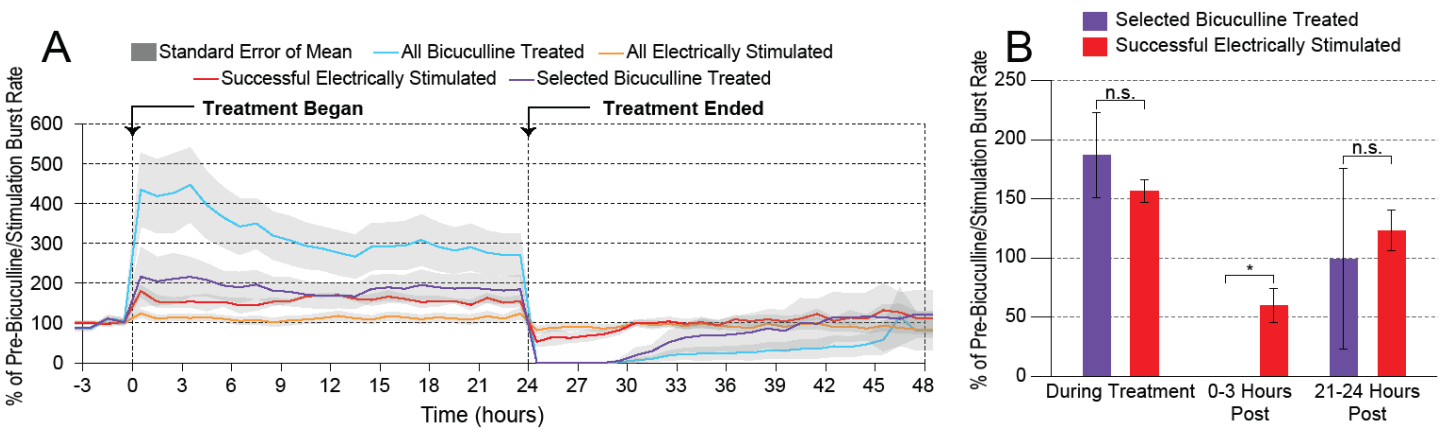

Figure 4. (A) Average percent of pre-bicuculline and pre-stimulation burst rates over time comparing all bicuculline treated cultures, all electrically stimulated cultures, successfully increased electrically stimulated cultures, and selected bicuculline treated cultures with comparable increases. Activity was normalized to controls and then averaged. Shaded areas represent SEM. (B) Normalized average activity for 24hrs during treatment (bicuculline or stimulation), and 3hrs or 24hrs following treatment. Electrically stimulated and bicuculline treated cultures with similar increases in burst rate were compared. Burst frequencies immediately following treatment were significantly different. Bars represent SEM. *$p<0.05$ (Bicuculline n=9, electrical n=14, successful electrical n=3, selected bicuculline n=3)

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